

10/088594

10100005.001198
JC10 Rec'd PCT/PTO 21 MAR 2002

TRANSMITTAL LETTER TO THE UNITED STATES
DESIGNATED/ELECTED OFFICE (DO/EO/US)
CONCERNING A FILING UNDER 35 U.S.C. 371

U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5)

10,088594

INTERNATIONAL APPLICATION NO
PCT/JP00/06471

INTERNATIONAL FILING DATE
21 September 2000

PRIORITY DATE CLAIMED
21 September 1999

TITLE OF INVENTION
NOVEL TRANSALDOLASE GENE

APPLICANT(S) FOR DO/EO/US
Masato Ikeda, et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. ☐ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
 - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☒ has been transmitted by the International Bureau.
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
 - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ have been transmitted by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☒ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 into English (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 into English (35 U.S.C. 371(c)(5)).

Items 11 to 20 below concern other document(s) or information included:

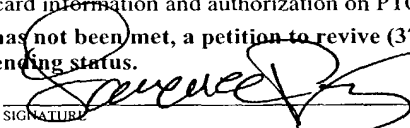
11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☒ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A **FIRST** preliminary amendment.
14. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
15. ☐ A substitute specification.
16. ☐ A change of power of attorney and/or address letter.
17. ☒ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
18. ☒ A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. ☒ Other items or information: Application Data Sheet; Form PCT/RO/101; Form PCT/ISA/210; Form PCT/IB/308; Form PCT/IB/332; Form PCT/IB345; Form PCT/IB/301; Form PCT/IB/304; Form PCT/IB/338 and Form PCT/IPEA/409.

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21. <input checked="" type="checkbox"/> The following fees are submitted:				CALCULATIONS	PTO USE ONLY
Basic National Fee (37 CFR 1.492(a)(1)-(5): Search Report has been prepared by the EP or JPO \$890.00 International preliminary examination fee paid to USPTO (37 CFR 1.492(a)(1)) \$710.00 No international preliminary examination fee paid to USPTO (37 CFR 1.492 (a)(1)) but international search fee paid to USPTO (37 CFR 1.492(a)(2)) \$740.00 Neither international preliminary examination fee (37 CFR 1.492(a)(1)) nor international search fee (37 CFR 1.492(a)(2)) paid to USPTO \$1,040.00 International preliminary examination fee paid to USPTO (37 CFR 1.492 (a)(4)) and all claims satisfied provisions of PCT Article 33(1)-(4) \$100.00 ENTER APPROPRIATE BASIC FEE AMOUNT =				\$890.00	
Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				\$	
Claims	Number Filed	Number Extra	Rate		
Total Claims	39 - 20 =	19	X \$18.00	\$342.00	
Independent Claims	4 - 3 =	1	X \$84.00	\$84.00	
Multiple dependent claim(s) (if applicable)			+ \$280.00	\$280.00	
TOTAL OF ABOVE CALCULATIONS =				\$1596.00	
<input type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.				+	\$
SUBTOTAL =				\$1596.00	
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f))				\$	
TOTAL NATIONAL FEE =				\$1596.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property +				\$40.00	
TOTAL FEES ENCLOSED =				\$1636.00	
				Amount to be:	
				refunded	\$
				charged	\$
a. <input checked="" type="checkbox"/> A check in the amount of \$ 1636.00 to cover the above fees is enclosed. b. <input type="checkbox"/> Please charge my Deposit Account No. _____ in the amount of \$ _____ to cover the above fees. A duplicate copy of this sheet is enclosed. c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 06-1205. A duplicate copy of this sheet is enclosed. d. <input type="checkbox"/> Fees are to be charged to a credit card. WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038. NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status. SEND ALL CORRESPONDENCE TO:					
Lawrence S. Perry FITZPATRICK, CELLA, HARPER & SCINTO 30 Rockefeller Plaza New York, NY 10112 Tel: (212) 218-2100 Fax: (212) 218-2200				SIGNATURE  Lawrence S. Perry NAME 31,865 REGISTRATION NUMBER	

JCO4 Rec'd PCT/PTO 11 JUN 2002

PCN



00005.001198

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

MASATO IKEDA, ET. AL.

Application No.: 10/088,594

I A. Filing Date: September 21, 2000

For: NOVEL TRANSALDOLASE
GENE

)
: Examiner. Not Yet Assigned

)
: Group Art Unit: Not Yet Assigned

)
: June 10, 2002

Commissioner for Patents
Washington, D.C. 20231

RESPONSE TO NOTIFICATION OF MISSING REQUIREMENTS
and
SUBMISSION OF CORRECTED COMPUTER READABLE FORM

Sir.

In response to the NOTIFICATION OF MISSING REQUIREMENTS
UNDER 35 U.S.C. §371 IN THE U.S. DESIGNATED/ELECTED OFFICE (DO/EO/US)
mailed May 20, 2002, Applicants submit herewith a computer readable form under 37
C.F.R. § 1.821(e). The content of the computer readable form and the Sequence Listing
filed herewith are the same. Also enclosed is copy of the NOTIFICATION OF MISSING
REQUIREMENTS UNDER 35 U.S.C. §371 IN THE U.S. DESIGNATED/ELECTED
OFFICE (DO/EO/US).

Also, enclosed is a check in the amount of \$18.00 to cover the required fee
for the additional claim.

The Assistant Commissioner is authorized to charge any additional fees
required to Deposit Account No 06-1205.

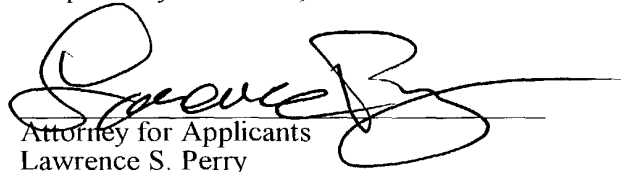
06/13/2002 GFREY1 00000089 10088594

01 FC:966

18.00 DP

Applicants' undersigned attorney may be reached in our New York office by telephone at (212) 218-2100. All correspondence should be directed to our below listed address.

Respectfully submitted,



Attorney for Applicants
Lawrence S. Perry
Registration No. 31,865

FITZPATRICK, CELLA, HARPER & SCINTO
30 Rockefeller Plaza
New York, New York 10112-3801
Facsimile: (212) 218-2200

NY_MAIN 266393v1

CRF Errors Corrected by the STIC Systems Branch

10032934-032102

PET10

Serial Number: 101088, 594A

CRF Processing Date: 7/22/02

Edited by: _____

Verified by: DC (STIC staff)

- ☐ Changed a file from non-ASCII to ASCII
- ☐ Changed the margins in cases where the sequence text was "wrapped" down to the next line.
- ☐ Edited a format error in the Current Application Data section, specifically:
ENTERED
- ☐ Edited the Current Application Data section with the actual current number. The number inputted by the applicant was ☐ the prior application data; or ☐ other _____.
- ☐ Added the mandatory heading and subheadings for "Current Application Data".
- ☐ Edited the "Number of Sequences" field. The applicant spelled out a number instead of using an integer.
- ☐ Changed the spelling of a mandatory field (the headings or subheadings), specifically:

- ☐ Corrected the SEQ ID NO when obviously incorrect. The sequence numbers that were edited were:

- ☒ Inserted or corrected a nucleic number at the end of a nucleic line. SEQ ID NO's edited:
1
- ☐ Corrected subheading placement. All responses must be on the same line as each subheading. If the applicant placed a response below the subheading, this was moved to its appropriate place.
- ☐ Inserted colons after headings/subheadings. Headings edited included:

- ☐ Deleted extra, invalid, headings used by an applicant, specifically:

- ☐ Deleted: ☐ non-ASCII "garbage" at the beginning/end of files; ☐ secretary initials/filename at end of file;
☐ page numbers throughout text; ☐ other invalid text, such as _____.
- ☒ Inserted mandatory headings, specifically: <220> in Seq. 3
- ☐ Corrected an obvious error in the response, specifically:

- ☐ Edited identifiers where upper case is used but lower case is required, or vice versa.
- ☐ Corrected an error in the Number of Sequences field, specifically:

- ☐ A "Hard Page Break" code was inserted by the applicant. All occurrences had to be deleted.
- ☐ Deleted **ending** stop codon in amino acid sequences and adjusted the "(A)Length:" field accordingly (error due to a PatentIn.bug). Sequences corrected: _____
- ☐ Other:

***Examiner: The above corrections must be communicated to the applicant in the first Office Action. DO NOT send a copy of this form.**

3/1/95

10/088594

IC10 Rec'd PCT/PTO 21 MAR 2002¹

00005.001198

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:)	
MASATO IKEDA, ET. AL.)	Examiner: Not Yet Assigned
)	Group Art Unit: Not Yet Assigned
Application No.: (National Phase of)	
PCT Application No. PCT/JP00/06471)	
filed September 21, 2000))	
Filed: Currently herewith)	
For. NOVEL TRANSALDOLASE)	
GENE)	March 20, 2002

Commissioner for Patents
Washington, D.C. 20231

PRELIMINARY AMENDMENT

Sir:

Prior to action on the merits, please amend the above-identified application
as follows:

IN THE SPECIFICATION

Please substitute the paragraph at page page 3, lines 17-19 with the
following replacement paragraph. A marked-up copy of this paragraph, showing the
changes made thereto, is attached.^{1/}

(3) A polypeptide comprising an amino acid sequence which is at least 60%
homologous to the amino acid sequence of SEQ ID NO: 1, and having transaldolase
activity.

^{1/} For the Examiner's convenience, bracketed deletions and underlined additions in
the marked-up copy are illustrated in bold.

4. (Amended) A DNA coding for the polypeptide of any one of claims 1 to 3, or the DNA of SEQ ID NO 2.

6. (Amended) A DNA which hybridizes with the DNA of claim 4 under stringent conditions, and codes for a polypeptide having transaldolase activity.

7 (Amended) A recombinant DNA obtained by ligating the DNA of claim 4 with a vector.

9 (Amended) A transformant in which one or more nucleotides have been substituted, deleted or inserted in the nucleotide sequence of the DNA carried by the transformant of claim 8 or in the nucleotide sequence of a DNA existing upstream the DNA and participating in transcription and translation, and of which the transaldolase activity is enhanced over that of the transformant not having undergone the substitution, deletion or insertion.

10. (Amended) The transformant according to claim 8, wherein the transformant has an ability to produce an aromatic amino acid or aromatic vitamin

12. (Amended) A transformant in which one or more nucleotides have been substituted, deleted or inserted in the nucleotide sequence of the DNA carried by the transformant of claim 8 or in the nucleotide sequence of a DNA existing upstream the DNA and participating in transcription and translation, and of which the transaldolase activity is lowered below that of the transformant not having undergone the substitution, deletion or insertion, or of which the transaldolase activity is lost.

13. (Amended) The transformant according to claim 8, wherein the transformant has an ability to produce a substance selected from L-histidine, riboflavin, nucleic acids and nucleic acid-associated substances

15. (Amended) A process for producing a polypeptide, which comprises culturing the transformant of claim 8 in a medium to thereby produce and accumulate the polypeptide in the culture, and recovering the polypeptide from the culture.

16. (Amended) A process for producing a saccharide having the dihydroxyacetone moiety of the ketose transferred into the aldose, which comprises allowing a ketose and an aldose to exist in an aqueous medium to coexist with an enzyme source selected from cells of the transformant of claim 9, a culture of the transformant or a processed product of the culture, to thereby produce and accumulate the saccharide in the aqueous medium, and recovering the saccharide from the aqueous medium.


REMARKS

The claims have been amended to correct their dependency and conformity with accepted U.S. practice and the specification has been changed to correct typographical errors. No new matter has been added.

Entry hereof is earnestly solicited

Applicants' undersigned attorney may be reached in our New York office by telephone at (212) 218-2100. All correspondence should continue to be directed to our below listed address

Respectfully submitted,


Lawrence S. Perry
Attorney for Applicants
Registration No. 31,865

FITZPATRICK, CELLA, HARPER & SCINTO
30 Rockefeller Plaza
New York, New York 10112-3801
Facsimile: (212) 218-2200

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VERSION WITH MARKINGS TO SHOW CHANGES MADE TO CLAIMS

3. (Amended) A [protein] polypeptide comprising an amino acid sequence which is at least 60 % homologous to the amino acid sequence of SEQ ID NO: 1, and having transaldolase activity.

4. (Amended) A DNA coding for the polypeptide of any one of claims 1 to 3, or the DNA of SEQ ID NO: 2

6. (Amended) A DNA which hybridizes with the DNA of claim 4 [or 5] under stringent conditions, and codes for a polypeptide having transaldolase activity.

7. (Amended) A recombinant DNA obtain[able]ed by ligating the DNA of [any one of] claim[s] 4 [to 6] with a vector.

9. (Amended) A transformant in which one or more nucleotides have been substituted, deleted or inserted in the nucleotide sequence of the DNA of [any one of claims 4 to 6] carried by the transformant of claim 8 or in the nucleotide sequence of a DNA existing upstream the DNA and participating in transcription and translation, and of which the transaldolase activity is enhanced over that of the transformant not having undergone the substitution, deletion or insertion.

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Attorney Docket No. 00005.001198

transformant or a processed product of the culture, to thereby produce and accumulate the
saccharide in the aqueous medium, and recovering the saccharide from the aqueous
medium.

Application No. National Phase of PCT Application No. PCT/JP00/06471
Attorney Docket No. 00005.001198

VERSION WITH MARKINGS TO SHOW CHANGES MADE TO SPECIFICATION

The paragraph at page 3, lines 17-19 have been amended as follows:

(3) A **[protein] polypeptide** comprising an amino acid sequence which is at least 60% homologous to the amino acid sequence of SEQ ID NO. 1, and having transaldolase activity.

The paragraph starting at page 13, line 15 and ending at page 14, line 7 has been amended as follows.

The host cells include microorganisms belonging to the genus Escherichia, Serratia, Bacillus, Brevibacterium, Corynebacterium, Microbacterium or Pseudomonas, including, for example, Escherichia coli XL1-Blue, Escherichia coli XL2-Blue, Escherichia coli DH1, Escherichia coli MC1000, Escherichia coli KY3276, Escherichia coli W1485, Escherichia coli JM109, Escherichia coli HB101, Escherichia coli No. 49, Escherichia coli W3110, Escherichia coli NY49, Escherichia coli G1698, Escherichia coli TB1, Serratia ficaria, Serratia fonticola, Serratia liquefaciens, Serratia marcescens, Bacillus subtilis, Bacillus [amyloliquefacines] amyloliquefaciens, Brevibacterium ammoniagenes, Brevibacterium immariophilum ATCC 14068, Brevibacterium saccharolyticum ATCC 14066, Brevibacterium flavum ATCC 14067, Brevibacterium lactofermentum ATCC 13869, Corynebacterium glutamicum ATCC 13032, Corynebacterium glutamicum ATCC 13869, Corynebacterium acetoacidophilum ATCC 13870, Microbacterium ammoniaphilum ATCC 15354, Pseudomonas putida and Pseudomonas sp. D-0110.

SPECIFICATIONNOVEL TRANSALDOLASE GENETECHNICAL FIELD

The present invention relates to a novel transaldolase gene, and to a polypeptide encoded by the gene, a recombinant DNA obtained by ligating the gene, a transformant carrying the recombinant DNA, and a process for producing the polypeptide, aromatic amino acids, aromatic vitamins, L-histidine, riboflavin, nucleic acids, nucleic acid-associated substances, novel saccharides and others by utilizing the transformant.

BACKGROUND ART

Transaldolase is an enzyme involved in pentose phosphate pathways, and plays an important role in biosynthesis and metabolism of aromatic compounds such as aromatic amino acids and aromatic vitamins, nucleic acid-associated substances such as purine-nucleotide and pyrimidine-nucleotide, as well as L-histidine, riboflavin and others [*Arch. Microbiol.*, 164, 324 (1995)]. Accordingly, a transaldolase gene and its gene products are useful as the target in breeding microorganisms for efficient fermentation and production of the metabolites.

As for transaldolase-encoding DNAs, an Escherichia coli-derived gene [Gene Bank Accession Number D13159], a Mycobacterium tuberculosis-derived gene [*Nature*, 393, 537

(1998)], and a Cinecococcus-derived gene [*Plant Mol. Biol.*, 30, 213 (1996)] were isolated; and their nucleotide sequences were determined.

It is reported that the productivity of aromatic compounds in Escherichia coli is increased when its transaldolase activity is increased (W098/18936).

However, for microorganisms belonging to the genus Corynebacterium that are widely used in amino acid fermentation of industrial importance, there is no report relating to the transaldolase gene and the enzyme encoded by the gene, and the nucleotide sequence of that gene is not known at all.

Regarding saccharide synthesis using transaldolase, an example is reported in which the enzyme is used for producing D-fructose from processed starch [*J. Am. Chem. Soc.*, 114, 6980 (1992)].

DISCLOSURE OF THE INVENTION

The object of the present invention is to provide a novel transaldolase gene, as well as a polypeptide encoded by the gene, a recombinant DNA obtained by ligating the gene, a transformant carrying the recombinant DNA, and a process for producing the polypeptide, aromatic amino acids, aromatic vitamins, L-histidine, riboflavin, nucleic acids, nucleic acid-associated substances, novel saccharides and others by using the transformant.

To achieve the object as mentioned above, the present inventors have used various techniques of DNA recombination and extensively studied the chromosomal genes of microorganisms belonging to the genus Corynebacterium. As a result, the present inventors have found that a transaldolase gene exists adjacent to the 3'-downstream site of a gene encoding transketolase, an enzyme differing from transaldolase, in a pentose phosphate pathway, and the present inventors have found the fact first. On the basis of this finding, the present invention has been completed. Specifically, the invention relates to the following subject matters (1) to (16):

(1) A polypeptide comprising the amino acid sequence of SEQ ID NO: 1.

(2) A polypeptide comprising the amino acid sequence of SEQ ID NO: 1 in which one or more amino acids have been substituted, deleted or added, and having transaldolase activity.

(3) A protein comprising an amino acid sequence which is at least 60 % homologous to the amino acid sequence of SEQ ID NO: 1, and having transaldolase activity.

(4) A DNA coding for the polypeptide of any one of above (1) to (3).

(5) A DNA comprising the nucleotide sequence of SEQ ID NO: 2.

(6) A DNA which hybridizes with the DNA of above (4) or (5) under stringent conditions, and codes for a polypeptide

having transaldolase activity.

(7) A recombinant DNA obtainable by ligating the DNA of any one of above (4) to (6) with a vector.

(8) A transformant carrying the recombinant DNA of above (7).

(9) A transformant in which one or more nucleotides have been substituted, deleted or inserted in the nucleotide sequence of the DNA of any one of above (4) to (6) carried by the transformant of above (8) or in the nucleotide sequence of a DNA existing upstream the DNA and participating in transcription and translation, and of which the transaldolase activity is enhanced over that of the transformant not having undergone the substitution, deletion or insertion.

(10) The transformant of above (8) or (9), wherein the transformant has an ability to produce an aromatic amino acid or aromatic vitamin.

(11) A process for producing an aromatic amino acid or aromatic vitamin, which comprises culturing the transformant of above (10) in a medium to thereby produce and accumulate in the culture the aromatic amino acid or aromatic vitamin, and recovering the aromatic amino acid or aromatic vitamin from the culture.

(12) A transformant in which one or more nucleotides have been substituted, deleted or inserted in the nucleotide sequence of the DNA of any one of above (4) to (6) carried by the transformant

(13) The transformant of above (8) or (12), wherein the transformant has an ability to produce a substance selected from L-histidine, riboflavin, nucleic acids and nucleic acid-associated substances.

(15) A process for producing the polypeptide of any one of above (1) to (3), which comprises culturing the transformant of above (8) in a medium to thereby produce and accumulate the polypeptide of any one of above (1) to (3) in the culture, and recovering the polypeptide from the culture.

(16) A process for producing a saccharide having the dihydroxyacetone moiety of the ketose transferred into the aldose, which comprises allowing a ketose and an aldose in an aqueous medium to coexist with an enzyme source selected from cells of

the transformant of above (8) or (9), a culture of the transformant or a processed product of the culture, to thereby produce and accumulate the saccharide in the aqueous medium, and recovering the saccharide from the aqueous medium.

The invention is described in detail hereinafter.

(1) Polypeptide of the present invention:

The polypeptide of the present invention has the amino acid sequence of SEQ ID NO: 1. Polypeptides having the amino acid sequence of SEQ ID NO: 1 in which one or more amino acids have been substituted, deleted or added, and having transaldolase activity is within the scope of the polypeptide of the present invention.

The polypeptide having the amino acid sequence which has been substituted, deleted or added, and having transaldolase activity can be obtained according to the site-specific mutation method described in *Molecular Cloning, A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press (1989) (hereinafter referred to as *Molecular Cloning*, 2nd Ed.), *Current Protocols in Molecular Biology*, John Wiley & Sons (1987-1997) (hereinafter referred to as *Current Protocols in Molecular Biology*), *Nucleic Acids Research*, 10, 6487 (1982), *Proc. Natl. Acad. Sci. USA*, 79, 6409 (1982), *Gene*, 34, 315 (1985), *Nucleic Acids Research*, 13, 4431 (1985), *Proc. Natl. Acad. Sci. USA*, 82, 488 (1985); for example, by introducing site-specific mutation into the DNA that codes for a polypeptide having the

The number of amino acids to be deleted, substituted or added is not specifically defined, but preferably from 1 to tens of amino acids, more preferably from 1 to a few amino acids are deleted, substituted or added. In order that the polypeptide of the present invention has transaldolase activity, it is desirable that its amino acid sequence is at least 60 %, generally at least 80 %, especially at least 95 % homologous to the amino acid sequence of SEQ ID NO: 1.

(2) DNA of the present invention:

DNAs that hybridize with the DNA of SEQ ID NO: 2 under stringent conditions are within the scope of the DNA of the present invention. The DNAs which hybridize with the DNA of SEQ ID NO: 2 under stringent conditions mean that it can be obtained through colony hybridization, plaque hybridization or southern hybridization using the DNA having the nucleotide sequence of SEQ ID NO: 2 or the internal fragment of the DNA as a probe. A specific example includes a DNA which can be identified through hybridization at 65°C on a filter on which a colony or

plaque-derived DNA or its fragment is fixed, in the presence of from 0.7 to 1.0 mol/liter of NaCl, followed by washing the filter at 65°C with an SSC solution of about 0.1 to 2-fold concentration (the SSC solution of 1-fold concentration comprises 150 mmol/l of sodium chloride and 15 mmol/l of sodium citrate).

The hybridization may be conducted according to the method described in, for example, the laboratory manual, *Molecular Cloning*, 2nd Ed. Specifically, the hybridizable DNA is, for example, a DNA having the nucleotide sequence which is at least 80 %, preferably at least 95 % homologous to the nucleotide sequence of SEQ ID NO: 2.

However, the DNA of the present invention does not include known DNAs that code for polypeptides having transaldolase activity.

The DNA of the present invention can be isolated from the chromosomal DNA of a microorganism that belongs to the genus Corynebacterium, according to the method mentioned hereinafter. As for the gene source, any microorganism belonging to the genus Corynebacterium can be used so long as it belongs to the genus Corynebacterium or Brevibacterium. Specific examples of such microorganisms are the following strains:

<u>Corynebacterium glutamicum</u>	ATCC 31833
<u>Corynebacterium glutamicum</u>	ATCC 13032
<u>Corynebacterium acetoacidophilum</u>	ATCC 13870

<u>Corynebacterium callunae</u>	ATCC 15991
<u>Corynebacterium herculis</u>	ATCC 13868
<u>Corynebacterium mellacecola</u>	ATCC 17965
<u>Corynebacterium lilium</u>	ATCC 15990
<u>Corynebacterium ammoniagenes</u>	ATCC 6872
<u>Brevibacterium immariophilum</u>	ATCC 14068
<u>Brevibacterium saccharolyticum</u>	ATCC 14066
<u>Brevibacterium thiogenitalis</u>	ATCC 19240
<u>Brevibacterium divaricutum</u>	ATCC 14020
<u>Brevibacterium flavum</u>	ATCC 14067
<u>Brevibacterium lactofermentum</u>	ATCC 13869

The chromosomal DNA of a microorganism belonging to genus Corynebacterium is extracted by using its culture, according to an ordinary method (for example, according to the method described in Japanese Published Unexamined Patent Application No. 126789/1983). The isolation of the DNA of the present invention from the chromosomal DNA comprises selecting a DNA which can complement the transketolase-deficient mutant [Appl. Microbiol. Biotechnol., 50, 375 (1998)] obtained as a shikimic acid-requiring mutant.

Specifically, the chromosomal DNA is cleaved with suitable restriction enzymes and ligated with a vector plasmid, then a transketolase gene-deficient mutant [e.g., Corynebacterium glutamicum TKT6 (FERMBP-6399)] is transformed with the resulting plasmid, and the transformant having recovered the requirement

(3) Production of Polypeptide of the present invention:

The polypeptide of the present invention can be produced according to the method described in *Molecular Cloning*, 2nd Ed. or *Current Protocols in Molecular Biology*, for example, by expressing the DNA of the present invention in host cells in the manner mentioned below.

A DNA fragment containing the part that codes for the polypeptide and having a suitable length is prepared. If desired, the nucleotide sequence coding for the polypeptide of the present invention is partly substituted with other nucleotides so as to be a codon most suitable for expression of the resulting DNA in host cells. The DNA is useful in efficient production of the polypeptide of the present invention.

The DNA fragment is inserted into the downstream site of the promoter of a suitable expression vector to construct a recombinant vector.

The recombinant vector is introduced into host cells suitable to the expression vector.

As a host cell, any cell can be used as long as it can express the intended gene, including bacteria, yeast cells, animal cells, insect cells and plant cells.

The expression vector shall be self-replicable in host cells or integrable with the chromosome therein, and shall have a promoter in the site in which the DNA coding for the polypeptide of the present invention can be transcribed.

In case where prokaryotes such as bacteria are used for

host cells, it is desirable that the recombinant vector containing the DNA coding for the polypeptide of the present invention is self-replicable in prokaryotes and comprises a promoter, a ribosome-binding sequence, the DNA of the present invention and a transcription termination sequence. If desired, the recombinant vector may contain a promoter control gene.

The expression vector includes, for example, pBTrp2, pBTac1, pBTac2 (all commercial products of Boehringer Mannheim), pKK233-2 (produced by Pharmacia), pSE280 (produced by Invitrogen), pGEMEX-1 (produced by Promega), pQE-8 (produced by QIAGEN), pKYP10 (Japanese Published Unexamined Patent Application No. 110600/1983), pKYP200 [*Agric. Biol. Chem.*, 48, 669 (1984)], pLSA1 [*Agric. Biol. Chem.*, 53, 277 (1989)], pGEL1 [*Proc. Natl. Acad. Sci. USA*, 82, 4306 (1985)], pBluescript II SK(-) (by Stratagene), pTrs30 [prepared from Escherichia coli JM109/pTrs30 (FERMBP-5407)], pTrs32 [prepared from Escherichia coli JM109/pTrs32 (FERM BP-5408)], pGHA2 [prepared from Escherichia coli IGHA2 (FERM B-400), Japanese Published Unexamined Patent Application No. 221091/1985], pGKA2 [prepared from Escherichia coli IGKA2 (FERM BP-6798), Japanese Published Unexamined Patent Application No. 221091/1985], pTerm2 (USP 4,686,191, 4,939,094, and 5,160,735), pSupex, pUB110, pTP5, pC194, pEG400 [*J. Bacteriol.*, 172, 2392 (1990)], pGEX (produced by Pharmacia), and pET system (produced by Novagen).

Any promoter may be used, so long as it is capable of

functioning in the host cells. For example, it is derived from Escherichia coli or phages, including trp promoter (P_{trp}), lac promoter, P_L promoter, P_R promoter and T7 promoter. Artificially-designed or modified promoters, such as $P_{trp} \times 2$ having two promoters in tandem, lacT7 promoter, and letI promoter.

A plasmid in which the distance between a Shine-Dalgarno sequence and an initiation codon is adjusted to an appropriate distance (e.g. 6 to 18 nucleotides) may be preferably used.

In the present invention, the recombinant vector does not always require a transcription termination sequence for expression of the DNA of the present invention. In the recombinant vector, however, it is desirable that a transcription termination sequence is just downstream the structural gene.

The host cells include microorganisms belonging to the genus Escherichia, Serratia, Bacillus, Brevibacterium, Corynebacterium, Microbacterium or Pseudomonas, including, for example, Escherichia coli XL1-Blue, Escherichia coli XL2-Blue, Escherichia coli DH1, Escherichia coli MC1000, Escherichia coli KY3276, Escherichia coli W1485, Escherichia coli JM109, Escherichia coli HB101, Escherichia coli No. 49, Escherichia coli W3110, Escherichia coli NY49, Escherichia coli GI698, Escherichia coli TB1, Serratia ficaria, Serratia fonticola, Serratia liquefaciens, Serratia marcescens, Bacillus subtilis, Bacillus amyloliquefacines, Brevibacterium ammoniagenes,

Brevibacterium immariophilum ATCC 14068, Brevibacterium saccharolyticum ATCC 14066, Brevibacterium flavum ATCC 14067, Brevibacterium lactofermentum ATCC 13869, Corynebacterium glutamicum ATCC 13032, Corynebacterium glutamicum ATCC 13869, Corynebacterium acetoacidophilum ATCC 13870, Microbacterium ammoniaphilum ATCC 15354, Pseudomonas putida and Pseudomonas sp. D-0110.

For introducing the recombinant vector into the host cells, any method of introducing DNA thereinto can be used, including the method by using calcium ions [*Proc. Natl. Acad. Sci. USA*, 69, 2110 (1972)], the protoplast method (Japanese Published Unexamined Patent Application No. 2483942/1988), and the methods described in *Gene*, 17, 107 (1982) and *Molecular & General Genetics*, 168, 111 (1979).

In case where yeast is used for host cells, the recombinant vector to be used in the case includes, for example, YEP13 (ATCC 37115), YEp24 (ATCC 37051), YCp50 (ATCC 37419), pHS19 and pHS15.

Any promoter may be used so long as it is capable of working in yeast cells, including, for example, promoters of genes participating in glycolysis such as hexose-kinase, as well as PH05 promoter, PGK promoter, GAP promoter, ADH promoter, gal 1 promoter, gal 10 promoter, heat-shock polypeptide promoter, MF α 1 promoter, and CUP 1 promoter.

The host cells include microorganisms belonging to the genus Saccharomyces, Schizosaccharomyces, Kluyveromyces,

For introducing the recombinant vector into the host cells, any method of introducing DNA into yeast cells can be used. For example, the electroporation method [*Methods Enzymol.*, 194, 182 (1990)], the spheroplast method [*Proc. Natl. Acad. Sci. USA*, 75, 1929 (1978)], the lithium acetate method [*J. Bacteriology*, 153, 163 (1983)], the method described in *Proc. Natl. Acad. Sci. USA*, 75, 1929 (1978) and the like, can be mentioned.

In case where animal cells are used for host cells, the expression vector to be used in the case includes, for example, pcDNAI, pcDM8 (produced by Funakoshi), pAGE107 [Japanese Published Unexamined Patent Application No. 22979/1991, *Cytotechnology*, 3, 133 (1990)], pAS3-3 (Japanese Published Unexamined Patent Application No. 227075/1990), pCDM8 [*Nature*, 329, 840 (1987)], pcDNAI/Amp (produced by Invitrogen), pREP4 (produced by Invitrogen), pAGE103 [*J. Biochem.*, 101, 1307 (1987)], and pAGE210.

Any promoter may be used as long as it is capable of functioning in animal cells, including, for example, the promoter of IE (immediate early) gene of cytomegalovirus (CMV), the early promoter of SV40 and the promoter of retrovirus, as well as metallothionein promoter, heat-shock promoter, and SR α promoter.

The enhancer of the IE gene of human CMV may be used together with the promoter.

The host cells include, for example, Namalwa cells that are human cells, COS cells that are monkey cells, CHO cells and HBT5637 cells that are Chinese hamster cells (Japanese Published Unexamined Patent Application No. 299/1988).

For introducing the recombinant vector into the animal cells, any method of introducing DNA into animal cells can be used. Forexample, the electroporation method [*Cytotechnology*, 3, 133 (1990)], the calcium phosphate method (Japanese Published Unexamined Patent Application No. 227075/1990), the lipofection method [*Proc. Natl. Acad. Sci. USA*, 84, 7413 (1987)], the method described in *Virology*, 52, 456 (1973) and the like, can be mentioned.

In case where insect cells are used for host cells, the polypeptide may be expressed, for example, according to the methods described in *Current Protocols in Molecular Biology; Baculovirus Expression Vectors*, A Laboratory Manual, W. H. Freeman and Company, New York (1992); and *Bio/Technology*, 6, 47 (1988).

That is, a recombinant gene-introduction vector and a Baculovirus are simultaneously introduced into insect cells to form a recombinant virus in the supernatant of the insect cell culture, and then insect cells are infected with the recombinant virus so as to express the polypeptide.

The gene-introduction vector to be used in the method includes, for example, pVL1392, pVL1393 and pBlueBacIII (all produced by Invitrogen).

The Baculovirus is, for example, *Autographa californica* nuclear polyhedrosis virus that infects insects of the family Barathra.

The insect cells include, for example, Spodopetera frugiperda oocytes, Sf9, Sf21 [*Baculovirus Expression Vectors*, A Laboratory Manual, W. H. Freeman and Company, New York (1992)], and Trichoplusia ni oocytes, High 5 (produced by Invitrogen).

For the simultaneously introduction of the recombinant gene-introduction vector and the Baculovirus into insect cells to prepare the recombinant virus, for example, a calcium phosphate method (Japanese Published Unexamined Patent Application No. 227075/1990), the lipofection method [*Proc. Natl. Acad. Sci. USA*, 84, 7413 (1987)] and the like, can be mentioned.

In case where plant cells are used for host cells, the expression vector includes, for example, Ti plasmid and tobacco mosaic virus vector.

Any promoter may be used, so long as it is capable of being expressed in plant cells, including, for example, 35S promoter of cauliflower mosaic virus (CaMV), and rice actin 1 promoter.

The plant cells for host cells include, for example, those of tobacco, potato, tomato, carrot, soybean, rape, alfalfa, rice, wheat, and barley.

For introducing the recombinant vector into such plant cells, any method of introducing DNA thereinto, including, for example, the method of introducing it into Agrobacterium (Japanese Published Unexamined Patent Application No. 140885/1984 and 70080/1985, WO94/00977), the electroporation method (Japanese Published Unexamined Patent Application No. 251887/1985), and the method by using a particle gun (gene gun) (Japanese Patent No. 2,606,856, 2,517,813) can be mentioned.

The gene expression may be conducted in a mode of direct expression, or alternatively, in a mode of secretion production or fused protein expression according to the method described in *Molecular Cloning*, 2nd Ed.

In case where the gene is expressed in yeast cells, animal cells, insect cells or plant cells, it gives a polypeptide with a saccharide or sugar chain added thereto.

The transformant of the present invention prepared in the manner as above is cultured in a medium, and the polypeptide of the present invention is produced and accumulated in the culture, and recovered from the culture.

For culturing the transformant of the present invention in a medium, any method as conventionally used for host cultivation in the art can be used.

In case where the transformant of the present invention is prepared by the use of prokaryotic cells such as Escherichia coli or eukaryotic cells such as yeast as a host cell, the medium

in which the transformant is cultured may be any natural or synthetic medium containing carbon sources, nitrogen sources and inorganic salts which can be assimilated by the transformant and in which the transformant can be efficiently cultured.

The carbon sources may be any ones which can be assimilated by the transformant, including, for example, carbohydrates such as glucose, fructose, sucrose, molasses containing them, starch or starch hydrolyzates; organic acids such as acetic acid, propionic acid and the like; and alcohols such as ethanol, propanol and the like.

The nitrogen sources include, for example, ammonia, ammonium salts of various inorganic acids and organic acids, such as ammonium chloride, ammonium sulfate, ammonium acetate, ammonium phosphate; other nitrogen-containing compounds; and peptone, meat extracts, yeast extracts, corn steep liquor, casein hydrolyzates, soy bean meal, soy bean meal hydrolyzates, various cells obtained by fermentation and their digested products.

The inorganic salts include, for example, potassium dihydrogenphosphate, dipotassium hydrogenphosphate, magnesium phosphate, magnesium sulfate, sodium chloride, ferrous sulfate, manganese sulfate, copper sulfate, and calcium carbonate.

Culturing the transformant in the medium is conducted under aerobic conditions, for example, in a mode of shaking culture or deep aeration stirring culture. The culturing temperature is preferably from 15 to 40°C, and the culturing period is

generally from 16 hours to 7 days. Preferably, the pH of the culture is from 3.0 to 9.0. For the pH control, any of inorganic or organic acids, alkali solutions, urea, calcium carbonate, ammonia or the like can be used.

If desired, antibiotics such as ampicillin and tetracycline may be added to the medium in which the transformant is cultured.

In case where microorganisms transformed with a recombinant vector having an inductive promoter are cultured, an inducer may be added to the medium, if desired. For example, when microorganisms transformed with a recombinant vector having lac promoter are cultured, isopropyl- β -D-thiogalactopyranoside may be added to the medium; and when microorganisms transformed with a recombinant vector having trp promoter are cultured, indole-acrylic acid may be added to the medium.

The medium in which the transformant prepared by the use of animal cells as a host cell is cultured may be any ordinary one, including, for example, RPMI1640 medium [*The Journal of the American Medical Association*, 199, 519 (1967)], Eagle's MEM medium [*Science*, 122, 501 (1952)], Dulbecco's modified MEM medium [*Virology*, 8, 396 (1959)], 199 medium [*Proceeding of the Society for the Biological Medicine*, 73, 1 (1950)], and those media with fetal calf serum therein.

Culturing the transformant in the medium is conducted generally at pH of from 6 to 8, at 30 to 40°C in the presence

of 5 % CO₂ for 1 to 7 days.

If desired, antibiotics such as kanamycin and penicillin may be added to the medium in which the transformant is cultured.

The medium in which the transformant prepared by the use of insect cells as a host cell is cultured may be any ordinary one, including, for example, TNM-FH medium (produced by PharMingen), Sf-900 IISFM medium (produced by Life Technologies), ExCell 400 and ExCell 405 [both produced by JRH Biosciences], Grace's Insect Medium [*Nature*, 195, 788 (1962)].

Culturing the transformant in the medium is conducted generally at pH of from 6 to 7, at 25 to 30°C for 1 to 5 days.

If desired, antibiotics such as gentamycin may be added to the medium in which the transformant is cultured.

The transformant cells prepared by the use of plant cells as a host cell may be cultured as they are, or after differentiated into plant cells or organs. The medium in which the transformant is cultured may be any ordinary one, including, for example, Murashige & Skoog (MS) medium, White medium, and those media with a plant hormone such as auxin or cytokinin therein.

Culturing the transformant in the medium is conducted generally at pH of from 5 to 9, at 20 to 40°C for 3 to 60 days.

If desired, antibiotics such as kanamycin and hygromycin may be added to the medium in which the transformant is cultured.

As described above, the transformant derived from microorganisms, animal cells or plant cells carrying a

The gene expression may be conducted in a mode of direct expression, or in a mode of secretion production or fused polypeptide expression according to the method described in *Molecular Cloning*, 2nd Ed.

In case where the polypeptide of the present invention is produced inside host cells or on the outer membrane of host cells, it can be positively secreted to the extracellular portion from the host cells, according to the method of Paulson, et al. [*J. Biol. Chem.*, 264, 17619 (1989)], the method of Lowe, et al. [*Proc. Natl. Acad. Sci., USA*, 86, 8227 (1989), *Genes Develop.*, 4, 1288 (1990)] or the methods described in Japanese Published Unexamined Patent Application No. 336963/1993 and WO94/23021.

That is, the polypeptide of the present invention can be positively secreted in the extracellular portion from the host cells, by expressing it in the form of a polypeptide containing the active site of the polypeptide of the present invention and

having a signal peptide upstream it, according to the genetic recombinant technology.

The yield of the polypeptide to be produced can be increased in a gene amplification system using a dihydrofolate reductase gene or the like, according to the method described in Japanese Published Unexamined Patent Application No. 227075/1990.

In addition, the gene-introduced animal or plant cells may be re-differentiated to construct gene-introduced animal individuals (transgenic non-human animals) or plant individuals (transgenic plants). Using these individuals, the polypeptide of the present invention may be produced.

In case where the transformant is an animal individual or plant individual, it may be raised or cultivated according to a conventional method to thereby produce and accumulate the intended polypeptide therein, and the polypeptide is recovered from the animal or plant individual.

For producing the polypeptide of the present invention in animal individuals, for example, an animal is transformed with the gene of the present invention introduced thereinto and the polypeptide is produced in the transformant animal according to known methods [*American Journal of Clinical Nutrition*, 63, 639S (1996), *American Journal of Clinical Nutrition*, 63, 627S (1996), *Bio/Technology*, 9, 830 (1991)].

For animal individuals, for example, the transgenic non-human animals carrying the DNA coding for the polypeptide

of the present invention are raised to thereby produce and accumulate the polypeptide in the animals, and the polypeptide is recovered from the animals. The site of the animals in which the polypeptide is produced and accumulated is, for example, milk (Japanese Published Unexamined Patent Application No. 309192/1988, eggs of the animals, and the like. Any promoter may be used so long as it is capable of being expressed in animals. Its preferred examples are mammary gland cell-specific promoters such as α -casein promoter, β -casein promoter, β -lactoglobulin promoter, whey acidic protein promoter, and the like.

For producing the polypeptide of the present invention in plant individuals, for example, a transgenic plant carrying the DNA coding for the polypeptide of the present invention is cultivated according to known methods [*Tissue Culture*, 20 (1994), *Tissue Culture*, 21 (1995), *Trends in Biotechnology*, 15, 45 (1997)] to thereby produce and accumulate the polypeptide in the plant, and the polypeptide is recovered from the plant.

For isolating and purifying the polypeptide produced by the transformant of the present invention, any conventional method for enzyme isolation and purification can be used. For example, when the polypeptide of the present invention is expressed in soluble forms inside the transformant cells, the cells are cultured, recovered from the culture by centrifuging the culture, then suspended in an aqueous buffer, and disrupted with an ultrasonic disrupter, French Press, Manton-Gaulin

homogenizer, Dynomill or the like to obtain a cell-free extract. The cell-free extract is centrifuged, and the resulting supernatant is purified through conventional enzyme isolation and purification. Specifically, for example, the supernatant is purified through solvent extraction, salting-out or desalting with sulfate ammonium or the like, precipitation with organic solvent, anion-exchange chromatography on resin such as diethylaminoethyl (DEAE)-Sephacrose or DIAION HPA-75 (produced by Mitsubishi Chemical Industries), or the like, cation-exchange chromatography on resin such as S-Sepharose FF (produced by Pharmacia) or the like, hydrophobic chromatography on resin such as butyl Sepharose or phenyl Sepharose, gel filtration through molecular sieve, affinity chromatography, chromatofocusing, or electrophoresis such as isoelectric focusing, or the like. The purification methods may be used either singly or as combined to obtain the intended pure product.

In case where the polypeptide is expressed as an inclusion body in the cells, the cells are similarly recovered, disrupted and centrifuged to give a precipitated fraction as the inclusion body including the polypeptide of the present invention. The thus-recovered insoluble polypeptide is solubilized with a protein denaturing agent. The solubilized solution is then diluted or dialyzed to thereby lower the concentration of the protein denaturing agent in the solution. Through the process, the solubilized polypeptide is renatured to have its own normal

tertiary structure. After thus processed, a pure product of the polypeptide is obtained through the same isolation and purification methods as in the above.

In case where the polypeptide of the present invention or the polypeptide derivative with a sugar chain added thereto is secreted outside from the cells, the polypeptide or the polypeptide derivative can be recovered in the culture supernatant. Specifically, the culture is centrifuged in the same manner as above to obtain the culture supernatant, and then a pure product of the polypeptide is obtained through the same isolation and purification methods as in the above from the culture supernatant.

The polypeptide thus obtained in the manner as above is, for example, the polypeptide having the amino acid sequence of SEQ ID NO: 1.

The polypeptide of the present invention can be produced through chemical synthesis of, for example, the Fmoc method (fluorenylmethyloxycarbonyl method) or the tBoc method (t-butyloxycarbonyl method). It may also be produced through chemical synthesis using peptide synthesizers such as those produced by Advanced ChemTech, Parkin-Elmer, Pharmacia, Protein Technology Instrument, Synthecell-Vega, PerSeptive, and Shimadzu.

(4) Method for producing substances using the DNA of the present invention:

In the transformants prepared in the above (3), the DNA

of the present invention or a DNA existing upstream the DNA of the present invention and participating the transcription and translation of the DNA is modified such that one or more nucleotides are deleted, substituted or added in its nucleotide sequence to thereby construct transformants that carry the thus-modified DNA (these are hereinunder referred to as transformant variants). From the transformant variants, transformant variants having enhanced transaldolase activity, or those having reduced or no transaldolase activity are selected. The nucleotides deletion, substitution and addition may be conducted in the known method (for example, described in *Molecular Cloning*, 2nd Ed., *Current Protocols in Molecular Biology*).

The transaldolase activity can be measured according to the process of Example (4) mentioned hereinafter.

That is, each transformant variant is cultured according to the method of above (3) to prepare a crude enzyme solution. The crude enzyme solution is added to a reaction solution [containing 40 mmol/l Tris (pH 7.6), 0.1 mmol/l diphosphopyridine, 2.8 mmol/l fructose 6-phosphate, 0.2 mmol/l erythrose 4-phosphate, 10 μ g of glycerol 3-phosphate dehydrogenase and triose phosphate isomerase mixture (produced by Boehringer Mannheim)] to make 1 ml, and the reaction is carried out at 25°C. The enzymatic activity of the transformant variant can be measured, by measuring the reduction in the absorbance at 340

According to the method as above, the transaldolase activity of transformant variants is measured, and the intended transformant variants having increased, reduced or lost transaldolase activity can be selected from all the transformant variants.

When using cells which can produce nucleic acid-associated substances such as purine-nucleotide and pyrimidine-nucleotide, L-histidine or riboflavin as a host cell, their ability to produce the substances can be increased, by lowering or deleting their transaldolase activity.

The transformant obtained in the manner as above is cultured in a medium to thereby produce and accumulate the intended product of aromatic amino acids, aromatic vitamins, L-histidine, riboflavin, purine-nucleotide, pyrimidine-nucleotide and other nucleic acid-associated substances, in the culture, and the product is isolated and purified from the culture according to known methods including concentration crystallization, activated charcoal treatment

and ion-exchange resin treatment [Isao Endo et al.'s Bioseparation Process Handbook, edited by the Chemical Engineering Society of Japan, published by Kyoritsu Publishing (1996)]. Through the process, the intended substance can be obtained efficiently.

Culturing the transformant may be conducted in the same manner as in the above (3) that indicates the method for culturing transformants for producing the polypeptide of the present invention.

(b) Production of novel saccharides:

A ketose and an aldose are allowed to exist in an aqueous medium containing, as an enzyme source, the transformant obtained according to the method mentioned above, a culture of the transformant or a processed product of the culture, to thereby produce and accumulate in the aqueous medium a saccharide having the dihydroxyacetone moiety of the ketose transferred into the aldose owing to the transaldolase activity of the enzyme source, and the saccharide is recovered from the aqueous medium.

The ketose includes, for example, sedoheptulose 7-phosphate, fructose 6-phosphate and the like; and the aldose includes, for example, erythrose 4-phosphate, glyceraldehyde 3-phosphate and the like.

The processed product of the transformant culture includes, for example, cultured cells; processed cells such as dried cells, lyophilized cells, surfactant-processed cells, enzyme-processed cells, ultrasonically-processed cells,

mechanically disrupted cells, solvent-processed cells; as well as enzyme products extracted from cultured cells, such as protein fractions of cultured cells, and immobilized products of cultured cells and processed cells.

The concentration of the enzyme source to be used in producing the saccharides of the present invention may be from 1 mU/liter to 1,000 U/liter, preferably from 10 mU/liter to 100 U/liter. One unit (U) is meant to indicate the activity of the enzyme source that produces 1 mmol of the saccharide at 37°C for 1 minute.

The aqueous medium to be used in producing the saccharides of the present invention includes, for example, water; buffers such as phosphates, carbonates, acetates, borates, citrates, Tris, or the like; alcohols such as methanol, ethanol, or the like; esters such as ethyl acetate, or the like; ketones such as acetone, or the like; and amides such as acetamide, or the like. The culture of the transformant serving as the enzyme source may also be used for the aqueous medium.

If desired, surfactant and organic solvent may be added to the system of producing the saccharides of the present invention. Any surfactant may be used so long as it is capable of promoting the production of galactose-containing saccharides, including, for example, nonionic surfactants such as polyoxyethylene-octadecylamine (e.g., Naimeen S-215 produced by Nippon Yushi); cationic surfactants such as

cetyltrimethylammonium bromide and alkyl dimethylbenzylammonium chlorides (e.g., Cation F2-40E produced by Nippon Yushi); anionic surfactants such as lauroyl sarcosinate; and tertiary amines such as alkyl dimethylamines (e.g., Tertiary Amine FB, produced by Nippon Yushi). One or more such surfactants may be used either singly or as combined. The surfactant concentration is usually from 0.1 to 50 g/l. The organic solvent includes, for example, xylene, toluene, aliphatic alcohols, acetone, and ethyl acetate. The solvent concentration is usually from 0.1 to 50 ml/liter.

The quantification of the saccharide formed in the aqueous medium according to the invention can be conducted, for example, with a Dionex's saccharide analyzer [*Anal. Biochem.*, 189, 151 (1990)].

The saccharides formed in the reaction mixture according to the present invention can be recovered by a conventional method, using activated charcoal or ion-exchange resin, or the like.

The method of the present invention makes it easy to produce saccharides which have heretofore been difficult to produce, and makes it possible to produce novel saccharides.

Examples of the present invention are described below, to which, however, the invention is not limited.

BEST MODES FOR CARRYING OUT THE INVENTION

(1) Acquisition of a transketolase-deficient mutant of

Corynebacterium glutamicum:

Corynebacterium glutamicum L22 [a lysozyme-sensitive mutant derived from a wild type strain ATCC 31833; R. Katsumata et al., *Proc. 4th Eur. Congr. Biotechnol.*, 4, 767 (1987)] was inoculated in 3ml of NB medium [containing 20 g of bouillon powder and 5 g of yeast extract in water 1 liter and having pH 7.2] and cultured therein at 30°C until OD₆₆₀ of the culture reached to 0.6.

After culturing, the cells were recovered through centrifugation, and washed once with 50 mmol/l Tris maleate buffer (pH 6.0), and subjected to a mutational treatment in 3 ml of the buffer containing 400 mg/l of NTG, at room temperature for 20 minutes. The treated cells were centrifuged and washed twice with the buffer, and then cultured in 3 ml of NB medium at 30°C for 1 hour.

The culture was diluted with physiological saline to 10⁻⁵ to 10⁻⁶, and 0.1 ml of the resulting dilution was spread on NB-agar medium [NB medium with 1.4 % agar, pH 7.2], and cultured thereon at 30°C for 2 days.

Each of the colonies grown on the agar medium were spread on minimal agar medium M1 [containing 10 g of glucose, 1 g of (NH₄)H₂PO₄, 0.2 g of KCl, 0.2 g of MgSO₄·7H₂O, 10 mg of FeSO₄·7H₂O, 0.2 mg of MnSO₄·4-6H₂O, 0.9 mg of ZnSO₄·7H₂O, 0.4 mg of CuSO₄·5H₂O, 0.09 mg of Na₂B₄O₇·10H₂O, 0.04 mg of (NH₄)₆Mo₇O₂₄·4H₂O, 50 mg of biotin, 2.5 mg of p-aminobenzoic acid, 1 mg of thiamin

hydrochloride and 16 g of agar in water 1 liter, and adjusted pH 7.2] and on M1 agar medium containing 50 mg/l shikimic acid, and cultured thereon at 30°C.

The colonies grown on the M1-agar medium containing 50 mg/l of shikimic acid, but not growing on the minimal agar medium M1 were separated as shikimic acid-requiring mutants. The thus-separated shikimic acid-requiring mutant were spread on M1-agar medium containing 50 mg/l of shikimic acid and onto the medium in which the glucose was substituted with ribose, and cultured thereon at 30°C.

The shikimic acid-requiring mutant grown on the M1-agar medium containing 50 mg/l of shikimic acid, but not growing on the medium containing ribose in place of glucose were separated as shikimic acid-requiring and ribose-non-assimilating mutants.

The thus-separated, shikimic acid-requiring and ribose-non-assimilating mutant cells were cultured in 40 ml of M1 medium containing 50 mg/l of shikimic acid, at 30°C for 24 hours. The cells were collected through centrifugation, ultrasonically disrupted, and again centrifuged to prepare a cell-free extract. The transketolase activity of the cell-free extract was determined as follows, using the cell-free extract as a crude enzyme solution.

The crude enzyme solution was added to a reaction solution [containing 50 mmol/l Tris (pH 7.5), 0.2 mmol/l NADH, 0.01 mmol/l

thiamin pyrophosphate, 1 mmol/l MgCl_2 , 0.5 mmol/l xylulose 5-phosphate, 0.5 mmol/l ribulose 5-phosphate, and 10 μg of a mixture of glycerol 3-phosphate dehydrogenase and triose phosphate isomerase (produced by Boehringer Mannheim)] to make 1.5 ml, and the reaction was carried out at 30°C.

The amount of glyceraldehyde 3-phosphate produced in the reaction mixture was determined by measuring the reduction in the absorbance at 340 nm of NADH in the reaction medium with spectrophotometer.

From the result of the measurement, a transketolase activity-deficient mutant TKT6, not being capable of producing glyceraldehyde 3-phosphate at all was selected, from the separated shikimic acid-requiring and ribose-non-assimilating mutants.

Corynebacterium glutamicum TKT6 has been deposited as FERM BP-6399 since June 30, 1998 with the International Patent Organism Depository National Institute of Advanced Industrial Science and Technology, Tsukuba Central 6, 1-1, Higashi 1-Chome Tsukuba-shi, Ibaraki-ken 305-8566 Japan (National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, 1-3, Higashi 1-chome, Tsukuba city, Ibaragi prefecture, 305-8566, Japan), under the Budapest Treaty.

(2) Cloning of DNA fragments containing transketolase gene and transaldolase gene:

For the source of the two genes, a chromosomal DNA of

Corynebacterium glutamicum ATCC 31833 was used; and for the recipient of the genes, the transketolase gene-deficient Corynebacterium glutamicum TKT6 (FERM BP-6399) obtained in Example 1 was used. For the vector, a plasmid pCSEK20 replicable in Corynebacterium glutamicum was used. The plasmid pCSEK20 comprises a replication origin of plasmid pCG2 derived from Corynebacterium glutamicum (Japanese Published Unexamined Patent Application No. 35197/1983), a spectinomycin and streptomycin-resistant gene of plasmid pCG4 derived from Corynebacterium glutamicum (Japanese Published Unexamined Patent Application No. 183799/1982) and a kanamycin-resistant gene of an conventionally-using-vector pGA22 [*J. Bacteriol.*, 140, 400 (1979)] for Escherichia coli [*Appl. Microbiol. Biotechnol.*, 51, 201 (1999)].

Culturing of Corynebacterium glutamicum ATCC 31833 and preparation of the chromosomal DNA from the culture was conducted according to the method described in Japanese Published Unexamined Patent Application No. 169785/1994. Plasmid pCSEK20 was isolated from the cultured cells of Corynebacterium glutamicum ATCC 31833 carrying it, according to the vector preparation method described in Japanese Published Unexamined Patent Application No. 169785/1994.

The cloning of the fragment containing transketolase gene and transaldolase gene from the chromosomal DNA of Corynebacterium glutamicum ATCC 31833 was conducted as follows.

The chromosomal DNA and the pCSEK20 plasmid DNA prepared as in the above, 1 µg each, were cleaved with EcoRI (5 units), and their fragments were ligated with a ligation kit (produced by Takara Shuzo). Using the thus-constructed plasmid, the shikimic acid-requiring, transketolase gene-deficient Corynebacterium glutamicum TKT6 (FERM BP-6399) was transformed as follows.

Corynebacterium glutamicum TKT6 was inoculated in 5 ml of NB medium, and cultured therein at 30°C for 1 day. The obtained seed culture (4 ml) was inoculated in 40 ml of SSM medium [containing 20 g of glucose, 10 g of (NH₄)₂SO₄, 3 g of urea, 1 g of yeast extract, 1 g of KH₂PO₄, 0.4 g of MgCl₂·6H₂O, 10 mg of FeSO₄·7H₂O, 0.2 mg of MnSO₄·4-6H₂O, 0.9 mg of ZnSO₄·7H₂O, 0.4 mg of CuSO₄·5H₂O, 0.09 mg of Na₂B₄O₇·10H₂O, 0.04 mg (NH₄)₆Mo₇O₂₄·4H₂O, 30 µg of biotin and 1 mg of thiamin hydrochloride in water 1 liter, and adjusted pH 7.2] containing 100 µg/ml shikimic acid, and cultured with shaking at 30°C until OD₆₆₀ of the culture reached to 0.6.

The cells were collected, and suspended in 10 ml of lysozyme-containing RCGP medium [containing 5 g of glucose, 5 g of casamino acid, 2.5 g of yeast extract, 1.5 g of KH₂PO₄, 0.41 g of MgCl₂·6H₂O, 10 mg of FeSO₄·7H₂O, 2 mg of MnSO₄·4-6H₂O, 0.9 mg of ZnSO₄·7H₂O, 0.04 mg of (NH₄)₆Mo₇O₂₄·4H₂O, 30 µg of biotin, 2 mg of thiamin hydrochloride, 135 g of disodium succinate and 30 g of polyvinylpyrrolidone (molecular weight 10,000) in water

1 liter] to have a cell concentration of about 10^9 cells/ml. The cell suspension was transferred into an L-shaped test tube and reacted therein with gently stirring at 30°C for 6 hours to obtain protoplast.

The thus-obtained 0.5 ml of the protoplast was put into a small test tube, centrifuged at $2,500 \times g$ for 5 minutes, re-suspended in 1 ml of TSMC buffer (10 mmol/l MgCl_2 , 30 mmol/l CaCl_2 , 50 mmol/l Tris, 400 mmol/l sucrose, pH 7.5), centrifuged and washed, and then re-suspended in 0.1 ml of TSMC buffer. The cell suspension was mixed with 100 μl of a 1/1 mixture of TSMC buffer of 2-fold concentration and the above-mentioned ligation mixture, followed by adding of 0.8 ml of 20 % PEG (6,000)-containing TSMC buffer, and further mixed. After 3 minutes, 2 ml of RCGP medium (pH 7.2) was added to the mixture, and centrifuged at $2,500 \times g$ for 5 minutes to remove the supernatant. The precipitated protoplast was suspended in 1 ml of RCGP medium. The cell suspension (0.2 ml) was spread on RCGP-agar medium (1.4 % agar-containing RCGP medium, pH 7.2) containing 200 $\mu\text{g/ml}$ kanamycin, and cultured thereon at 30°C for 10 days.

The colonies grown on the agar medium were scraped up, centrifuged and washed twice with physiological saline, and suspended in 1 ml of physiological saline. The cell suspension was again spread on minimal agar medium M1 [containing 10 g of glucose, 1 g of $(\text{NH}_4)_2\text{HPO}_4$, 0.2 g of KCl , 1 g of KH_2PO_4 , 0.2 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 10 mg of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 mg of $\text{MnSO}_4 \cdot 4-6\text{H}_2\text{O}$, 0.9

mg of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4 mg of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.09 mg of $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$, 0.04 mg of $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, 50 μg of biotin, 2.5 mg of p-aminobenzoic acid, 1 mg of thiamin hydrochloride and 16 g of agar in water 1 liter, and adjusted pH 7.2] containing 20 $\mu\text{g}/\text{ml}$ kanamycin, and cultured thereon at 30°C for 3 days. Through the process, a kanamycin-resistant and shikimic acid-non-requiring transformant was selected.

From transformants, plasmid DNAs were isolated according to the vector preparation method described in Japanese Published Unexamined Patent Application No. 169785/1994. A plasmid was obtained from one strain of the transformant, and named pCTK60. DNA fragments cleaved with restriction enzymes were analyzed through agarose gel electrophoresis, which confirmed that the plasmid has a structure of about 7.6 kb EcoRI DNA fragment inserted into the EcoRI site of pCSEK20. Through subcloning and complementation test, it was found that at least a transketolase gene exist on about 4.1 kb XhoI-EcoRI DNA fragment contained in the EcoRI DNA fragment.

(3) Sequencing of XhoI-EcoRI DNA fragment:

From the plasmid having approximately 4.1 kb XhoI-EcoRI DNA fragment, the DNA fragment was recovered according to a conventional method. The DNA fragment and a vector pUC19 (produced by Takara Shuzo) were cleaved with various restriction enzymes, and the vector DNA fragment was ligated with the decomposed DNA fragment by using a T4DNA ligase. Escherichia

The strains grown on the selective medium were inoculated in LB medium containing ampicillin to a final concentration of 100 µg/ml, and cultured therein at 30°C for 12 hours. From the cultured cells, a plasmid was isolated through alkali-SDS method (*Molecular Cloning*, 2nd Ed.).

The nucleotide sequence was analyzed with a sequence-analyzing soft, GENETYX MAC ATSQ 3.0 by Software Development.

As a result, it was found that two open reading frames exist in the nucleotide sequence of the 4.1 kb XhoI-EcoRI DNA

fragment.

The primary structure of the amino acid sequence estimated from the nucleotide sequence of the DNA fragment was compared with that of the amino acid sequence of transketolase and transaldolase of Mycobacterium tuberculosis which is taxonomically similar to the genus Corynebacterium, and it was confirmed that the open reading frame existing at 373rd to 2472nd in the nucleotide sequence of SEQ ID NO: 3 is a transketolase gene, and the open reading frame existing at 2643rd to 3722nd is a transaldolase gene. The amino acid sequence estimated from the open reading frame of the transaldolase gene is shown by SEQ ID NO: 1; and the nucleotide sequence thereof is shown by SEQ ID NO:2.

(4) Determination of transketolase activity and transaldolase activity:

The XhoI-EcoRI DNA fragment of approximately 4.1 kb was, after its both ends had been repaired to be blunt according to the conventional method, inserted into the SmaI site of a vector pCG116 replicable in Corynebacterium glutamicum [Appl. Microbiol. Biotechnol., 51, 201 (1999)] to construct a recombinant plasmid pHTK65. Corynebacterium glutamicum ATCC 31833 was transformed with the recombinant plasmid, and the transketolase and transaldolase activity of the transformant was measured. The transketolase activity of the transformant was measured according to the method described in Japanese

Published Unexamined Patent Application No. 169785/1994; and the transaldolase activity thereof was measured as follows.

A crude enzyme solution was added to a reaction solution [containing 40 mmol/l Tris (pH 7.6), 0.1 mmol/l diphosphopyridine, fructose 6-phosphate 2.8 mmol/l, 0.2 mmol/l erythrose 4-phosphate, 10 µg of a mixture of glycerol 3-phosphate dehydrogenase and triose phosphate isomerase mixture (produced by Boehringer Mannheim)] to make 1 ml, and the reaction was carried out at 25°C. The amount of glyceraldehyde 3-phosphate produced in the reaction mixture was quantified by measuring the reduction in the absorbance at 340 nm of the reaction medium by using a spectrophotometer. Both of the transketolase activity and the transaldolase activity of the pHTK65-carrying transformant increased at least 5 times more than those of ATCC31833, when those of ATCC 31833 per unit protein weight and unit time is defined to be one.

INDUSTRIAL APPLICABILITY

The novel transaldolase gene of the present invention and its nucleotide sequence information, as well as the polypeptide encoded by the gene and its amino acid sequence information make it possible to modify the transaldolase activity of microorganisms belonging to the genus Corynebacterium which are widely used in amino acid fermentation of industrial importance, to breed the microorganisms for amino acid fermentation. In

addition, they also make it possible to breed strain having high transaldolase activity and are useful in stereospecific carbon-carbon bond forming reaction for production of saccharides and their derivatives.

of which the transaldolase activity is enhanced over that of the transformant not having undergone the substitution, deletion or insertion.

10. The transformant according to claim 8 or 9, wherein the transformant has an ability to produce an aromatic amino acid or aromatic vitamin.

11. A process for producing an aromatic amino acid or aromatic vitamin, which comprises culturing the transformant of claim 10 in a medium to thereby produce and accumulate in the culture the aromatic amino acid or aromatic vitamin, and recovering the aromatic amino acid or aromatic vitamin from the culture.

12. A transformant in which one or more nucleotides have been substituted, deleted or inserted in the nucleotide sequence of the DNA of any one of claims 4 to 6 carried by the transformant of claim 8 or in the nucleotide sequence of a DNA existing upstream the DNA and participating in transcription and translation, and of which the transaldolase activity is lowered below that of the transformant not having undergone the substitution, deletion or insertion, or of which the transaldolase activity is lost.

13. The transformant according to claim 8 or 12, wherein the transformant has an ability to produce a substance selected from L-histidine, riboflavin, nucleic acids and nucleic acid-associated substances.

14. A process for producing a substance selected from

L-histidine, riboflavin, nucleic acids and nucleic acid-associated substances, which comprises culturing the transformant of claim 13 in a medium to thereby produce and accumulate the substance in the culture, and recovering the substance from the culture.

15. A process for producing the polypeptide of any one of claims 1 to 3, which comprises culturing the transformant of claim 8 in a medium to thereby produce and accumulate the polypeptide of any one of claims 1 to 3 in the culture, and recovering the polypeptide from the culture.

16. A process for producing a saccharide having the dihydroxyacetone moiety of the ketose transferred into the aldose, which comprises allowing a ketose and an aldose to exist in an aqueous medium to coexist with an enzyme source selected from cells of the transformant of claim 8 or 9, a culture of the transformant or a processed product of the culture, to thereby produce and accumulate the saccharide in the aqueous medium, and recovering the saccharide from the aqueous medium.

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[JP/JP]; 〒755-8501 山口県宇部市大字藤曲2548番地
協和醸酵工業株式会社 宇部工場内 Yamaguchi (JP).

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(71) 出願人 (米国を除く全ての指定国について): 協和醸酵工業株式会社 (KYOWA HAKKO KOGYO CO., LTD.) [JP/JP]; 〒100-8185 東京都千代田区大手町一丁目6番1号 Tokyo (JP).

添付公開書類:

(72) 発明者; および

— 国際調査報告書

(75) 発明者/出願人 (米国についてのみ): 池田正人 (IKEDA, Masato) [JP/JP]. 高野 裕 (TAKANO, Yutaka) [JP/JP]. 鎌田 望 (KAMADA, Nozomu) [JP/JP]; 〒194-8533 東京都町田市旭町3丁目6番6号 協和醸酵工業株式会社 東京研究所内 Tokyo (JP). 中野哲郎 (NAKANO, Tetsuo)

— 明細書とは別に規則 13 の 2 に基づいて提出された生物材料の寄託に関する表示。

2 文字コード及び他の略語については、定期発行される各 PCT ガゼットの巻頭に掲載されている「コードと略語のガイダンスノート」を参照。

(54) Title: NOVEL TRANSALDOLASE GENE

(54) 発明の名称: 新規トランスアルドラーゼ遺伝子

(57) Abstract: Attempts are made to provide a novel transaldolase gene; a polypeptide encoded by this gene; a recombinant DNA obtained by integrating this gene; a microorganism carrying this recombinant DNA; and a process for producing an aromatic amino acid, an aromatic vitamin, L-histidine, riboflavin, a nucleic acid, a nucleic acid-associated substance, a novel saccharide, etc. by using the above microorganism. As the results of extensive studies, a novel transaldolase gene is isolated from chromosomal DNA of a microorganism belonging to the genus *Corynebacterium* as a DNA fragment complementary to the requirement for shikimic acid of a transketolase defective variant obtained as a variant with the requirement for shikimic acid belonging to the genus *Corynebacterium*. Further, a recombinant DNA containing this gene is constructed and transferred into a host microorganism, thereby achieving the objects as described above.

[続葉有]

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FOR PATENT COOPERATION TREATY APPLICATION**

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled NOVEL TRANSALDOLASE GENE

the specification of which was filed as PCT International Application No. PCT/JP00/06471 on 21.09.00 and was amended under PCT Article 19 on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, § 119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) on which priority is claimed:

Country	Application No.	Filed (Day/Mo./Yr.)	Priority Claimed (Yes/No)
Japan	266548/99	21 September 1999	Yes

I hereby appoint the practitioners associated with the firm and Customer Number provided below to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith, and direct that all correspondence be addressed to the address associated with that Customer Number:

FITZPATRICK, CELLA, HARPER & SCINTO

Customer Number: 05514

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FOR PATENT COOPERATION TREATY APPLICATION
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1-00

Full Name of Sole or First Inventor Masato Ikeda
Inventor's Signature Masato Ikeda
Date March 8, 2002 Citizen/Subject of JAPAN
Residence Tokyo, Japan SPX
Post Office Address c/o Tokyo Research Laboratories, KYOWA HAKKO KOGYO CO., LTD.
6-6, Asahi-machi 3-chome, Machida-shi, Tokyo 194-8533 Japan

2-00

Full Name of Second Joint Inventor, if any Yutaka Takano
Inventor's Signature Yutaka Takano
Date March 8, 2002 Citizen/Subject of JAPAN
Residence Tokyo, Japan SPX
Post Office Address c/o Tokyo Research Laboratories, KYOWA HAKKO KOGYO CO., LTD.
6-6, Asahi-machi 3-chome, Machida-shi, Tokyo 194-8533 Japan

3-00

Full Name of Third Joint Inventor, if any Tetsuo Nakano
Inventor's Signature Tetsuo Nakano
Date March 11, 2002 Citizen/Subject of JAPAN
Residence Ube-shi, Japan SPX
Post Office Address c/o Ube Plant, KYOWA HAKKO KOGYO CO., LTD.
2548, Ohaza-Fujimagari, Ube-shi, Yamaguchi 755-8501 Japan

4-00

Full Name of Fourth Joint Inventor, if any Nozomu Kamada
Inventor's Signature Nozomu Kamada
Date March 8, 2002 Citizen/Subject of JAPAN
Residence Tokyo, Japan SPX
Post Office Address c/o Tokyo Research Laboratories, KYOWA HAKKO KOGYO CO., LTD.
6-6, Asahi-machi 3-chome, Machida-shi, Tokyo 194-8533 Japan

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gag	gca	tcc	tcc	atc	gct	ggc	acc	cag	cag	ctg	ggc	aac	ctc	atc	gtg	987
Glu 190	Ala	Ser	Ser	Ile	Ala 195	Gly	Thr	Gln	Gln	Leu 200	Gly	Asn	Leu	Ile	Val 205	
ttc	tgg	gat	gac	aac	cgc	atc	tcc	atc	gaa	gac	aac	act	gag	atc	gct	1035
Phe	Trp	Asp	Asp	Asn 210	Arg	Ile	Ser	Ile	Glu 215	Asp	Asn	Thr	Glu 220	Ile	Ala	
ttc	aac	gag	gac	gtt	gtt	gct	cgt	tac	aag	gct	tac	ggc	tgg	cag	acc	1083
Phe	Asn	Glu	Asp 225	Val	Val	Ala	Arg	Tyr 230	Lys	Ala	Tyr	Gly	Trp 235	Gln	Thr	
att	gag	gtt	gag	gct	ggc	gag	gac	gtt	gca	gca	atc	gaa	gct	gca	gtg	1131
Ile	Glu 240	Val	Glu	Ala	Gly	Glu	Asp 245	Val	Ala	Ala	Ile	Glu 250	Ala	Ala	Val	
gct	gag	gct	aag	aag	gac	acc	aag	cga	cct	acc	ttc	atc	cgc	gtt	cgc	1179
Ala	Glu	Ala	Lys	Lys	Asp	Thr	Lys	Arg	Pro	Thr	Phe	Ile	Arg	Val	Arg	

SECRET

acc atc atc ggc ttc cca gct cca acc atg atg aac acc ggt gct gtg 1227
Thr Ile Ile Gly Phe Pro Ala Pro Thr Met Met Asn Thr Gly Ala Val
270 275 280 285

cac ggt gct gct ctt ggc gca gct gag gtt gca gca acc aag act gag 1275
 His Gly Ala Ala Leu Gly Ala Ala Glu Val Ala Ala Thr Lys Thr Glu
 290 295 300

cct gga ttc gat cct gag gct cac ttc gcg atc gac gat gag gtt atc 1323
Leu Gly Phe Asp Pro Glu Ala His Phe Ala Ile Asp Asp Glu Val Ile

305 310 315

gct cac acc cgc tcc ctc gca gag cgc gct gca cag aag aag gct gca 1371
Ala His Thr Arg Ser Leu Ala Glu Arg Ala Ala Gln Lys Lys Ala Ala
320 325 330

tgg	cag	gtc	aag	ttc	gat	gag	tgg	gca	gct	gcc	aac	cct	gag	aac	aag	1419
Trp	Gln	Val	Lys	Phe	Asp	Glu	Trp	Ala	Ala	Ala	Asn	Pro	Glu	Asn	Lys	
	335					340					345					

gct ctg ttc gat cgc ctg aac tcc cgt gag ctt cca gcg ggc tac gct 1467
Ala Leu Phe Asp Arg Leu Asn Ser Arg Glu Leu Pro Ala Gly Tyr Ala
350 355 360 365

gac gag ctc cca aca tgg gat gca gat gag aag ggc gtc gca act cgt 1515
Asp Glu Leu Pro Thr Trp Asp Ala Asp Glu Lys Gly Val Ala Thr Arg
370 375 380

aag gct tcc gag gct gca ctt cag gca ctg ggc aag acc ctt cct gag 1563
Lys Ala Ser Glu Ala Ala Leu Gln Ala Leu Gly Lys Thr Leu Pro Glu
385 390 395

ctg	tgg	ggc	ggt	tcc	gct	gac	ctc	gca	ggt	tcc	aac	aac	acc	gtg	atc	1611
Leu	Trp	Gly	Gly	Ser	Ala	Asp	Leu	Ala	Gly	Ser	Asn	Asn	Thr	Val	Ile	
		400					405					410				

aag ggc tcc cct tcc ttc ggc cct gag tcc atc tcc acc gag acc tgg 1659
Lys Gly Ser Pro Ser Phe Gly Pro Glu Ser Ile Ser Thr Glu Thr Trp
415 420 425

tct	gct	gag	cct	tac	ggc	cgt	aac	ctg	cac	ttc	ggt	atc	cgt	gag	cac	1707
Ser	Ala	Glu	Pro	Tyr	Gly	Arg	Asn	Leu	His	Phé	Gly	Ile	Arg	Glu	His	
430					435					440					445	

gct atg gga tcc atc ctc aac ggc att tcc ctc cac ggt ggc acc cgc 1755
Ala Met Gly Ser Ile Leu Asn Gly Ile Ser Leu His Gly Gly Thr Arg
450 455 460

cca Pro	tac Tyr	ggt Gly	gga Gly 465	acc Thr	ttc Phe	ctc Leu	atc Ile	ttc Phe 470	tcc Ser	gac Asp	tac Tyr	atg Met	cgt Arg 475	cct Pro	gca Ala	1803
gtt Val	cgt Arg	ctt Leu 480	gca Ala	gct Ala	ctc Leu	atg Met	gag Glu 485	acc Thr	gac Asp	gct Ala	tac Tyr	tac Tyr 490	gtc Val	tgg Trp	acc Thr	1851
cac His	gac Asp 495	tcc Ser	atc Ile	ggt Gly	ctg Leu	ggc Gly 500	gaa Glu	gat Asp	ggc Gly	cca Pro	acc Thr 505	cac His	cag Gln	cct Pro	gtt Val	1899
gaa Glu 510	acc Thr	ttg Leu	gct Ala	gcg Ala	ctg Leu 515	cgc Arg	gcc Ala	atc Ile	cca Pro	ggt Gly 520	ctg Leu	tcc Ser	gtc Val	ctg Leu	cgt Arg 525	1947
cct Pro	gca Ala	gat Asp	gcg Ala	aat Asn 530	gag Glu	acc Thr	gcc Ala	cag Gln	gct Ala 535	tgg Trp	gct Ala	gca Ala	gca Ala	ctt Leu 540	gag Glu	1995
tac Tyr	aag Lys	gaa Glu	ggc Gly 545	cct Pro	aag Lys	ggt Gly	ctt Leu	gca Ala 550	ctg Leu	acc Thr	cgc Arg	cag Gln	aac Asn 555	gtt Val	cct Pro	2043
gtt Val	ctg Leu	gaa Glu 560	ggc Gly	acc Thr	aag Lys	gag Glu	aag Lys 565	gct Ala	gct Ala	gaa Glu	ggc Gly	gtt Val 570	cgc Arg	cgc Arg	ggt Gly	2091
ggc Gly	tac Tyr 575	gtc Val	ctg Leu	gtt Val	gag Glu	ggt Gly 580	tcc Ser	aag Lys	gaa Glu	acc Thr	cca Pro 585	gat Asp	gtg Val	atc Ile	ctc Leu	2139
atg Met 590	ggc Gly	tcc Ser	ggc Gly	tcc Ser	gag Glu 595	gtt Val	cag Gln	ctt Leu	gca Ala	gtt Val 600	aac Asn	gct Ala	gcg Ala	aaa Lys	gct Ala 605	2187
ctg Leu	gaa Glu	gct Ala	gag Glu	ggc Gly 610	gtt Val	gca Ala	gct Ala	cgc Arg	gtt Val 615	gtt Val	tca Ser	gtt Val	cct Pro	tgc Cys 620	atg Met	2235
gat Asp	tgg Trp	ttc Phe	cag Gln 625	gag Glu	cag Gln	gac Asp	gca Ala	gag Glu 630	tac Tyr	atc Ile	gag Glu	tcc Ser	gtt Val 635	ctg Leu	cct Pro	2283
gca Ala	gct Ala	gtg Val 640	acc Thr	gct Ala	cgt Arg	gtg Val	tct Ser 645	gtt Val	gaa Glu	gct Ala	ggc Gly 650	atc Ile	gca Ala	atg Met	cct Pro	2331
tgg Trp	tac Tyr	cgc Arg	ttc Phe	ttg Leu	ggc Gly	acc Thr	cag Gln	ggc Gly	cgt Arg	gct Ala	gtc Val	tcc Ser	ctt Leu	gag Glu	cac His	2379

655	660	665	
ttc ggt gct tct gcg gat tac cag acc ctg ttt gag aag ttc ggc atc			2427
Phe Gly Ala Ser Ala Asp Tyr Gln Thr Leu Phe Glu Lys Phe Gly Ile			
670	675	680	685
acc acc gat gca gtc gtg gca gcg gcc aag gac tcc att aac agt			2472
Thr Thr Asp Ala Val Val Ala Ala Ala Lys Asp Ser Ile Asn Ser			
	690	695	700
taattgccct gctgttttta gcttcaaccc ggggcagtat gattctccgg aattttattg			2532
ccccgggttg ttgttggttaa tcggtacaaa ggggtcttaag cacatccctt acttgcctgc			2592
tctccttgag cacagttcaa gaacaattct tttaaggaaa atttagtttc atg tct			2648
			Met Ser
			1
cac att gat gat ctt gca cag ctc ggc act tcc act tgg ctc gac gac			2696
His Ile Asp Asp Leu Ala Gln Leu Gly Thr Ser Thr Trp Leu Asp Asp			
	5	10	15
ctc tcc cgc gag cgc att act tcc ggc aat ctc agc cag gtt att gag			2744
Leu Ser Arg Glu Arg Ile Thr Ser Gly Asn Leu Ser Gln Val Ile Glu			
	20	25	30
gaa aag tct gta gtc ggt gtc acc acc aac cca gct att ttc gca gca			2792
Glu Lys Ser Val Val Gly Val Thr Thr Asn Pro Ala Ile Phe Ala Ala			
	35	40	50
gca atg tcc aag ggc gat tcc tac gac gct cag atc gca gag ctc aag			2840
Ala Met Ser Lys Gly Asp Ser Tyr Asp Ala Gln Ile Ala Glu Leu Lys			
	55	60	65
gcc gct ggc gca tct gtt gac cag gct gtt tac gcc atg agc atc gac			2888
Ala Ala Gly Ala Ser Val Asp Gln Ala Val Tyr Ala Met Ser Ile Asp			
	70	75	80
gat gtt cgc aat gct tgt gat ctg ttc acc ggc atc ttc gag tcc tcc			2936
Asp Val Arg Asn Ala Cys Asp Leu Phe Thr Gly Ile Phe Glu Ser Ser			
	85	90	95
aac ggc tac gac ggc cgc gtg tcc atc gag gtt gac cca cgt atc tct			2984
Asn Gly Tyr Asp Gly Arg Val Ser Ile Glu Val Asp Pro Arg Ile Ser			
	100	105	110
gct gac cgc gac gca acc ctg gct cag gcc aag gag ctg tgg gca aag			3032
Ala Asp Arg Asp Ala Thr Leu Ala Gln Ala Lys Glu Leu Trp Ala Lys			
	115	120	130
gtt gat cgt cca aac gtc atg atc aag atc cct gca acc cca ggt tct			3080

Val	Asp	Arg	Pro	Asn	Val	Met	Ile	Lys	Ile	Pro	Ala	Thr	Pro	Gly	Ser		
				135					140					145			
ttg	cca	gca	atc	acc	gac	gct	ttg	gct	gag	ggc	atc	agc	gtt	aac	gtc		3128
Leu	Pro	Ala	Ile	Thr	Asp	Ala	Leu	Ala	Glu	Gly	Ile	Ser	Val	Asn	Val		
			150					155					160				
acc	ttg	atc	ttc	tcc	gtt	gct	cgc	tac	cgc	gag	gtc	atc	gct	gcg	tac		3176
Thr	Leu	Ile	Phe	Ser	Val	Ala	Arg	Tyr	Arg	Glu	Val	Ile	Ala	Ala	Tyr		
			165				170					175					
atc	gag	gga	atc	aag	cag	gca	gct	gca	aac	ggc	cac	gac	gta	tcc	aag		3224
Ile	Glu	Gly	Ile	Lys	Gln	Ala	Ala	Ala	Asn	Gly	His	Asp	Val	Ser	Lys		
	180					185					190						
atc	cac	tct	gtg	gct	tcc	ttc	ttc	gtc	tcc	cgc	gtc	gac	gtt	gag	atc		3272
Ile	His	Ser	Val	Ala	Ser	Phe	Phe	Val	Ser	Arg	Val	Asp	Val	Glu	Ile		
	195				200					205					210		
gac	aag	cgc	ctc	gag	gca	atc	gga	tcc	gat	gag	gct	ttg	gct	ctg	cgc		3320
Asp	Lys	Arg	Leu	Glu	Ala	Ile	Gly	Ser	Asp	Glu	Ala	Leu	Ala	Leu	Arg		
			215						220					225			
ggc	aag	gca	ggc	gtt	gcc	aac	gct	cag	cgc	gct	tac	gct	gtg	tac	aag		3368
Gly	Lys	Ala	Gly	Val	Ala	Asn	Ala	Gln	Arg	Ala	Tyr	Ala	Val	Tyr	Lys		
			230					235					240				
gag	ctt	ttc	gac	gcc	gcc	gag	ctg	cct	gaa	ggt	gcc	aac	act	cag	cgc		3416
Glu	Leu	Phe	Asp	Ala	Ala	Glu	Leu	Pro	Glu	Gly	Ala	Asn	Thr	Gln	Arg		
		245					250					255					
cca	ctg	tgg	gca	tcc	acc	ggc	gtg	aag	aac	cct	gcg	tac	gct	gca	act		3464
Pro	Leu	Trp	Ala	Ser	Thr	Gly	Val	Lys	Asn	Pro	Ala	Tyr	Ala	Ala	Thr		
	260					265					270						
ctt	tac	gtt	tcc	gag	ctg	gct	ggt	cca	aac	acc	gtc	aac	acc	atg	cca		3512
Leu	Tyr	Val	Ser	Glu	Leu	Ala	Gly	Pro	Asn	Thr	Val	Asn	Thr	Met	Pro		
	275				280					285					290		
gaa	ggc	acc	atc	gac	gct	gtt	ctg	gaa	ctg	ggc	aac	ctg	cac	ggt	gac		3560
Glu	Gly	Thr	Ile	Asp	Ala	Val	Leu	Glu	Leu	Gly	Asn	Leu	His	Gly	Asp		
				295				300						305			
acc	ctg	tcc	aac	tcc	gcg	gca	gaa	gct	gac	gct	gtg	ttc	tcc	cag	ctt		3608
Thr	Leu	Ser	Asn	Ser	Ala	Ala	Glu	Ala	Asp	Ala	Val	Phe	Ser	Gln	Leu		
			310					315					320				
gag	gct	ctg	ggc	gtt	gac	ttg	gca	gat	gtc	ttc	cag	gtc	ctg	gag	acc		3656
Glu	Ala	Leu	Gly	Val	Asp	Leu	Ala	Asp	Val	Phe	Gln	Val	Leu	Glu	Thr		
		325					330					335					

gag ggt gtg gac aag ttt gtt gct tct tgg agc gaa ctg ctt gag tcc 3704
Glu Gly Val Asp Lys Phe Val Ala Ser Trp Ser Glu Leu Leu Glu Ser
340 345 350

atg gaa gct cgc ctg aag tagaatcagc acgctgcatc agtaacggcg 3752
Met Glu Ala Arg Leu Lys
355 360

acatgaaatc gaattagttc gatcttatgt ggccggttaca catctttcat taaagaaagg 3812
atcgtgacgc taccatcgtg agcacaaaca cgacccccctc cagctggaca aaccactgc 3872
gcgacccgca ggataaacga ctcccccgca tcgctggccc ttccggcatg gtgatcttcg 3932
gtgtcactgg cgacttggct cgaaggaagc tgctccccgc catttatgat ctagcaaacc 3992
gcggtattgct gccccagga ttctcgttgg taggttacgg ccgccgcgaa tgggtccaaag 4052
aagactttga aaaatacgta cgcgatgccg caagtgctgg tgctcgtacg gaattc 4108